



ELSEVIER

Veterinary Parasitology 103 (2002) 309–313

veterinary  
parasitology

www.elsevier.com/locate/vetpar

# Survival of nonsporulated *Toxoplasma gondii* oocysts under refrigerator conditions

D.S. Lindsay<sup>a,\*</sup>, B.L. Blagburn<sup>b</sup>, J.P. Dubey<sup>c</sup>

<sup>a</sup> Department of Biomedical Sciences and Pathobiology, Virginia–Maryland College of Veterinary Medicine, Centre for Molecular Medicine and Infectious Diseases, Virginia Tech., 1410 Prices Fork Road, Blacksburg, VA 24061-0442, USA

<sup>b</sup> Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849-5519, USA

<sup>c</sup> United States Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Parasite Biology, Epidemiology and Systematics Laboratory, Beltsville, MD 20705-2350, USA

Received 18 July 2001; received in revised form 21 August 2001; accepted 21 August 2001

## Abstract

*Toxoplasma gondii* oocysts are excreted nonsporulated in the feces of the cats into the environment. These oocysts must undergo sporulation to become infectious. Little is known about the factors that influence sporulation of *T. gondii* oocysts. The present study examined the survival of nonsporulated oocysts under refrigerated conditions over 11-week observation period. Microscopic examination of oocysts indicated that no visible development occurred under refrigerator conditions. The nonsporulated oocysts retained their ability to sporulate when placed at room temperature. The numbers of visually viable appearing oocysts decreased over time. Some oocysts in all samples were infectious for mice despite being refrigerated for up to an 11 weeks before undergoing sporulation. Results indicate that nonsporulated oocysts can survive in the environment for at least 3 months and retain their ability to become infectious when placed under appropriate conditions. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** *Toxoplasma gondii*; Epidemiology; Oocyst; Zoonosis; Sporulation

## 1. Introduction

*Toxoplasma gondii* has been identified by the President's Food Safety Initiative as a major foodborne pathogen of concern (FDA, 1997, 1999). It is a ubiquitous, facultatively heterogenous, apicomplexan protozoan parasite of warm-blooded animals including humans (Frenkel et al., 1970). Domestic cats and other felines are the only known definitive

\* Corresponding author. Tel.: +1-540-231-6302; fax: +1-540-231-3426.

E-mail address: lindsayd@vt.edu (D.S. Lindsay).

hosts, while man is a common intermediate host. Cats can excrete millions of environmentally resistant oocysts in their feces. It has been documented that sporulated *T. gondii* oocysts can survive in the environment for 1.5 years (Frenkel et al., 1975) and for 4.5 years at 4 °C (Dubey, 1998). There were approximately 57 million cats in the United States in 1991 (Patronek, 1998). The prevalence of antibodies to *T. gondii* is about 58% in free-roaming cats and 37% in pet cats (Dubey, 1994). Ingestion of unwashed raw fruits or vegetables is associated with an increased risk of maternally acquired toxoplasmosis (Kapperud et al., 1996). Contact with the soil is also associated with an increased risk of maternal toxoplasmosis (Decavalas et al., 1990) and *T. gondii* oocysts have been isolated from soil obtained from gardens (Coutinho et al., 1982). Humans can become infected when they ingest food, water, or other consumable products that have been contaminated with oocysts (Sulzer et al., 1986; Isaac-Renton et al., 1998; Aramini et al., 1999). Raw or undercooked meat is also a source of *T. gondii* for humans (Dubey and Beattie, 1988). It is presently impossible to determine if a person was initially infected by ingestion of *T. gondii* oocysts or through consumption of infected meat containing tissue cysts.

The environmental resistance of *T. gondii* oocysts may depend on the stage of sporulation of oocysts. For example, exposure of oocysts at 37 °C for 24 h was lethal for nonsporulated *T. gondii* oocysts (Dubey et al., 1970) whereas sporulated oocysts survived for at least 32 days at 35 °C and 9 days at 40 °C. The present study was done to determine the effects of storage under refrigerator conditions (4 °C) on sporulation and viability of nonsporulated *T. gondii* oocysts.

## 2. Materials and methods

### 2.1. *T. gondii* oocysts and oocyst examinations

Two *T. gondii* naive cats were fed mouse brains containing tissue cysts of the VEG strain of *T. gondii*. Cats were housed and infected in a cat colony at the United States Department of Agriculture, Parasite Biology, Epidemiology and Systematics Laboratory, Beltsville, MD as described by Dubey (1995). Feces containing nonsporulated oocysts were collected, placed in the refrigerator at 4 °C, and sent on cool packs to the Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL. A 20 g sample was mixed in a minimal amount of 2% sulfuric acid solution and kept at 4 °C in a refrigerator to serve as the source of future study material. Six hundred oocysts were examined at 40× under a light microscope to determine if sporulation had occurred during collection and transport. Additionally,  $5 \times 10^5$  of these source oocysts were fed to each of 2 ICR female mice and  $5 \times 10^5$  were subcutaneously injected into each of 2 ICR female mice. Additional portions of the feces were mixed in 2% sulfuric acid solution, strained through a tea strainer, and placed at a depth of 5 mm in petri dishes. These cultures were incubated at room temperature (estimated to be 22–25 °C) for 1 and 2 days and then processed and  $5 \times 10^5$  oocysts fed to each of 2 ICR female mice.

Subsamples were taken from the refrigerated fecal sample containing *T. gondii* oocysts at 6, 7, 8, 9, 10, and 11 weeks. Two hundred oocysts were examined at 40× under a light microscope from each sample prior to it being placed for sporulation and oocysts were

classified as sporulated or not sporulated. Samples were processed as above and allowed to sporulate for 1 week at room temperature in petri dishes. After the samples had been allowed to sporulate for 1 week, they were examined and the numbers of oocysts in the 2-celled stage or oocysts which appeared degenerate were recorded for 100 oocysts. It is difficult to see sporozoites in *T. gondii* oocysts at 40× and thus the oocysts were categorized as being in the 2-celled stage and not as sporulated. The remaining sample was placed in a 15 ml screw top centrifuge tube and stored at 4 °C until used for mouse inoculation.

## 2.2. Bioassay for infective *T. gondii* in mice

Female ICR, 25–30 g mice were housed in groups of two and used for oocyst infectivity studies. As previously stated, mice were each fed 5000 *T. gondii* oocysts. This number was based on the numbers of oocysts which were in the 2-celled stage after sporulation. The count does not include any degenerated appearing oocysts. Mice were examined daily for clinical signs of toxoplasmosis. Surviving mice were bled from the retro-orbital plexus and killed 4 weeks post-feeding of oocysts. Their sera were examined for antibodies to *T. gondii* in an indirect immunofluorescent antibody test (IFAT) at a dilution of 1:50. The brain from each mouse was examined for *T. gondii* tissue cysts as a squash preparation (Dubey and Beattie, 1988).

## 3. Results

None of the 600 oocysts examined from the source feces contained oocysts that had developed to the 2-celled stage. Mice fed or subcutaneously injected with oocysts from this source did not develop toxoplasmosis, and have IFAT antibodies or tissue cysts in their brains at necropsy. None of the mice fed with oocysts that had been sporulated at room temperature for 1 day developed toxoplasmosis, had IFAT antibodies, or tissue cysts in their brains at necropsy. Mice fed with oocysts which had been sporulated at room temperature for 2 days did not develop toxoplasmosis. One of these mice developed an IFAT titer and had tissue cysts in its brains at necropsy, while the other mouse in this group remained negative by IFAT and by brain examination.

Table 1

Results of storing *T. gondii* oocysts at 4 °C on their subsequent sporulation at room temperature

| Time stored | 2-Cell stage/degenerate stage <sup>a</sup> | No. mice inoculated/No. positive <sup>b</sup> |
|-------------|--|---|
| 6 weeks     | 30/70                                      | 2/2   |
| 7 weeks     | 22/78                                      | 2/2   |
| 8 weeks     | 12/88                                      | 2/2   |
| 9 weeks     | 4/96                                       | 2/2   |
| 10 weeks    | 6/94                                       | 2/2   |
| 11 weeks    | 1/99                                       | 2/2   |

<sup>a</sup> Number of oocysts in the 2-celled stage/number of oocysts which appeared to be degenerated and not capable of sporulation.

<sup>b</sup> Mice were considered positive if tissue cysts or antibodies to *T. gondii* were present at necropsy.

No oocysts removed from the refrigerator had sporulated or developed to the 2-cell stage prior to being placed at room temperature. Table 1 presents the results of oocyst characterizations after sporulation for 1 week at room temperature and the results of mouse feeding studies. One mouse in the group that received 5000 oocysts from the 8 week culture and one mouse in the group that received 5000 oocysts from the 11 week culture died. *T. gondii* was found in the tissues of both of these mice. All other mice fed *T. gondii* oocysts survived the length of the study and had tissue cysts in their brains at necropsy.

#### 4. Discussion

Surprisingly little is known about the sporulation of *T. gondii* oocysts. Sporulation of *T. gondii* oocysts occurs in the environment outside the body of the feline definitive host and like other coccidial parasites is dependent on temperature and moisture (Dubey et al., 1970). Sporulation is asynchronous and some oocysts will be sporulated before others.

We did not observe any oocysts in the 2-celled stage from the source cat feces and none of the mice fed or inoculated with these oocysts became infected. This proves that our source oocysts had not undergone sporulation in the shipping process. Our study demonstrated that no development of nonsporulated *T. gondii* oocysts occurs at 4 °C because all oocysts were still in the single-cell stage prior to being placed in petri dishes for sporulation, confirming earlier observations by Dubey et al. (1970). Our microscopic observations (Table 1) indicate that some nonsporulated oocysts lose the ability to sporulate after storage under refrigerator conditions for 6–11 weeks. Results of our mouse feeding studies demonstrated that infectious oocysts were present at all observation times. This demonstrates that although there was a decrease in the ability of refrigerated oocysts to sporulate, some oocysts in the populations remained viable and were able to sporulate and be infectious for mice.

Dubey et al. (1970) reported that infectious oocysts were present by 24 h at 25 °C (room temperature), by 5 days at 15 °C, and by 21 days at 11 °C. In our characterization of our source oocysts used in this study, we did not demonstrate orally infectious oocysts until 48 h. This may reflect differences in *T. gondii* strains used, differences in methods used to sporulate oocysts or differences in ambient room temperatures between the present study and that of Dubey et al. (1970).

Because of the public health importance of *T. gondii* and the recent recognition of water-borne outbreaks of human toxoplasmosis (Isaac-Renton et al., 1998; Aramini et al., 1999), it is important to learn more about the biology of the oocyst in the environment. Studies on nonsporulated oocysts and sporulated oocysts survival in water and other potential environmental sources are needed.

#### References

- Aramini, J.J., Stephen, C., Dubey, J.P., Engelstoft, C., Schwantje, H., Ribble, C.S., 1999. Potential contamination of drinking water with *Toxoplasma gondii* oocysts. *Epidemiol. Infect.* 122, 305–315.
- Coutinho, S.G., Lobo, R., Dutra, G., 1982. Isolation of *Toxoplasma* from the soil during an outbreak of toxoplasmosis in a rural area in Brazil. *J. Parasitol.* 68, 66–868.

- Decavalas, G., Papapetropoulou, M., Giannoulaki, E., Tzigounis, V., Kondakis, X.G., 1990. Prevalence of *Toxoplasma gondii* antibodies in gravidas and recently aborted women and study of risk factors. *Eur. J. Epidemiol.* 6, 223–226.
- Dubey, J.P., 1994. Toxoplasmosis. *J. Am. Vet. Med. Assoc.* 205, 1593–1598.
- Dubey, J.P., 1995. Duration of immunity to shedding of *Toxoplasma gondii* oocysts by cats. *J. Parasitol.* 81, 410–415.
- Dubey, J.P., 1998. *Toxoplasma gondii* oocyst survival under defined temperatures. *J. Parasitol.* 84, 862–865.
- Dubey, J.P., Beattie, C.P., 1988. *Toxoplasmosis of Animals and Man*. CRC Press, Boca Raton, pp. 1–40.
- Dubey, J.P., Miller, N.L., Frenkel, J.K., 1970. The *Toxoplasma gondii* oocyst from cat feces. *J. Exp. Med.* 132, 636–662.
- FDA, 1997. Food Safety from Farm to Table: A National Food Safety Initiative. Food Safety and Inspection Service, Centers for Disease Control and Prevention, FDA, 1997.
- FDA, 1999. Healthy People 2000: Status Report Food Safety Objectives. Food Safety and Inspection Service, Centers for Disease Control and Prevention, FDA, 1999.
- Frenkel, J.K., Dubey, J.P., Miller, N.L., 1970. *Toxoplasma gondii* in cats: fecal stages identified as coccidian oocysts. *Science* 167, 893–896.
- Frenkel, J.K., Ruiz, A., Chinchilla, M., 1975. Soil survival of *Toxoplasma* oocysts in Kansas and Costa Rica. *Am. J. Trop. Med. Hyg.* 24, 439–443.
- Isaac-Renton, J., Bowie, W.R., King, A., Irwin, G.S., Ong, C.S., Fung, C.P., Shokeir, M.O., Dubey, J.P., 1998. Detection of *Toxoplasma gondii* oocysts in drinking water. *Appl. Env. Microbiol.* 64, 2278–2280.
- Kapperud, G., Jenum, P.A., Stray-Pedersen, B., Melby, K.K., Eskild, A., Eng, J., 1996. Risk factors for *Toxoplasma gondii* infection in pregnancy. Results of a prospective case-control study in Norway. *Am. J. Epidemiol.* 144, 405–412.
- Patronek, G.J., 1998. Free-roaming and feral cats—their impact on wildlife and human beings. *J. Am. Vet. Med. Assoc.* 212, 218–226.
- Sulzer, A.J., Franco, E.L., Takafuji, E., Benenson, M., Walls, K.W., Greenup, R.L., 1986. An oocyst-transmitted outbreak of toxoplasmosis: patterns of immunoglobulin G and M over one year. *Am. J. Trop. Med. Hyg.* 35, 290–296.